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Bromocresol Purple, a Non-Specific Colour Reagent for the Determination of Serum Albumin

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Summary: The determination of serum albumin with the dye reagent bromocresol purple was investigated.

We found that bromocresol purple is not a specific reagent for albumin, but that serum proteins in the α-, β-, and γ-globulin fractions also react with this dye. Similar results were obtained for bromocresol green.

Furthermore there is a great difference in absorbance between human and bovine albumin solutions (having the same concentration) when using the bromocresol purple reagent. Most probably this is a result of the difference in interaction of the reagent with the substrate.

Although the standard curves with the bromocresol purple method were more linear (especially at higher albumin concentrations) than those obtained with the bromocresol green method, we still prefer the bromocresol green technique to the bromocresol purple technique, because its results are more comparable with those of other methods.

Moreover in quality control and calibration procedures, strong deviations from the recommended values are obtained when non-human sera are analyzed with the bromocresol purple method.

For reasons of accuracy more specific methods are preferable.

Bromkresolpurpur, ein nicht-spezifisches Farbreagenz zur Bestimmung von Serum-Albumin

Zusammenfassung: Die Bestimmung von Serum-Albumin mit dem Farbreagenz Bromkresolpurpur wurde untersucht. Wir fanden, daß Bromkresolpurpur kein spezifisches Reagenz für Albumin ist und Serumproteine in der α-, β- und γ-Globulinfraktion auch mit diesem Farbstoff reagieren. Ähnliche Ergebnisse wurden mit Bromkresolgrün erhalten. Es besteht ferner ein großer Unterschied in der Absorption von Albumin des Menschen und des Rindes gleicher Konzentration, wenn Bromkresolpurpur als Reagenz benutzt wird. Sehr wahrscheinlich beruht dies auf unterschiedlichen Wechselwirkungen des Reagenz mit dem Protein.

Obwohl die Standardkurven mit der Bromkresolpurpur-Methode – besonders bei höheren Albuminkonzentrationen – geradliniger sind als die mit der Bromkresolgrün-Methode, ziehen wir unter Berücksichtigung des Vergleichs der Ergebnisse mit denen anderer Methoden doch die Bromkresolgrün-Methode der Bromkresolpurpur-Methode vor.

Darüberhinaus werden bei Verwendung von Seren, die nicht vom Menschen stammen, in Qualitätskontroll- und Standardisierungsverfahren mit der Bromkresolpurpur-Methode starke Abweichungen von den angegebenen Werten erhalten.

Hinsichtlich der Genauigkeit sind spezifischere Methoden vorzuziehen.

Introduction

The determination of serum albumin with bromocresol green is a well known procedure (1–7). Unfortunately the use of this colour reaction has some disadvantages. The reaction is not a specific one because other serum proteins and some drugs (4) also give a colour. Upon standing, the reagent partly precipitates, causing turbidity (4).

Recently a new colour reagent, bromocresol purple was documented (4, 8, 9, 10). The authors indicated that this reagent was preferred to bromocresol green because of the specificity of bromocresol purple for serum al-

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bumin. No reactions were observed with other serum proteins. Only large amounts of bilirubin would hinder the determination (4).

In order to see whether this method could be used for our serum albumin determinations we started to investigate this bromocresol purple technique. In some quality control experiments (National Foundation of Quality Control of Clinical Chemical Hospital Laboratories, Nijmegen) we observed very large deviations from the mean values.

The measurements were repeated with new control sera (with the same composition) but the differences still remained. We therefore undertook a detailed investigation of this determination.

Materials and Methods

Reagents

The working bromocresol green solution was prepared according to the method of *McPherson* (2).

The working bromocresol purple solution was prepared according to the method of *Pinnell* (4). We used 2 ml instead of 1 ml of the bromocresol purple reagent (see Results and l.c. (10)).

Procedure (bromocresol purple and bromocresol green methods)

To 10 ml of the dye reagent was added 50 µl of serum. After mixing, the colour change was read (against a reagent blank) as soon as possible on a Beckman Spectrophotometer, Model 26, at 603 nm for the bromocresol purple method, and at 635 nm for the bromocresol green method. In both cases a human albumin standard and/or a standard curve was used to estimate the amount of serum albumin. Human and bovine albumin were obtained from Hoechst-Behring.

Electrophoresis of serum proteins

Electrophoresis, followed by staining with the dye reagents bromocresol purple and bromocresol green

About 3.0 µl of serum was applied to a cellogel cellulose acetate strip (Chemetron, Italy) and was run for 45 min at a constant potential difference of 250 V at room temperature. After electrophoresis, fixation of the proteins and deacetylation of the strips were performed in one step by soaking the strips for 5 to 10 minutes in a mixture of 10 g NaOH + 500 ml distilled water + 430 ml ethanol (960 ml/l). During the process the strips shrank, losing their original size. This shrinking process is complete after about 5 minutes. The strips were steeped in a buffer with pH of about 3–4 to change the pH to that of the dye reagent, then immersed in the dye reagent for about 15 minutes.

Routine protein electrophoresis

About 0.12 µl of serum was applied to the cellulose acetate strips (Sartorius Membrane filter) and run for 30 min at a constant potential difference of 250 V at room temperature. Next the strips were stained in a bath of amido black for 5 min., then decolorized in a mixture of acetic acid and methanol (100 ml + 900 ml) for 5 minutes.

After 3 min in a transparency bath (mixture of 700 ml dioxane + 300 ml *iso*-butanol), the strips were dried at 100 °C for 10 minutes.

Evaluation

The scans were carried out on a Clifford Densicomp, model 445.

M-Partigen Immunodiffusion Plates for Albumin Determination

The manufacturer's instructions for the M-Partigen Immunodiffusion Plates (Hoechst-Behring) were followed. The precipitate circles were measured with a Measuring Viewer for Immunoanalysis (Behringwerke) and the albumin content read from the standard curve.

Total protein determination

The total protein determinations were carried out on an Auto Analyzer II continuous flow system (Technicon Instruments) using the biuret reaction.

Results

The standard curves of some albumin solutions are shown in figure 1.

Only the results for albumin solutions in distilled water are given, because the same values (within the experimental error) were found for albumin in physiological salt solution (9 g sodium chloride in one liter of water). Although bovine albumin gives a straight standard curve

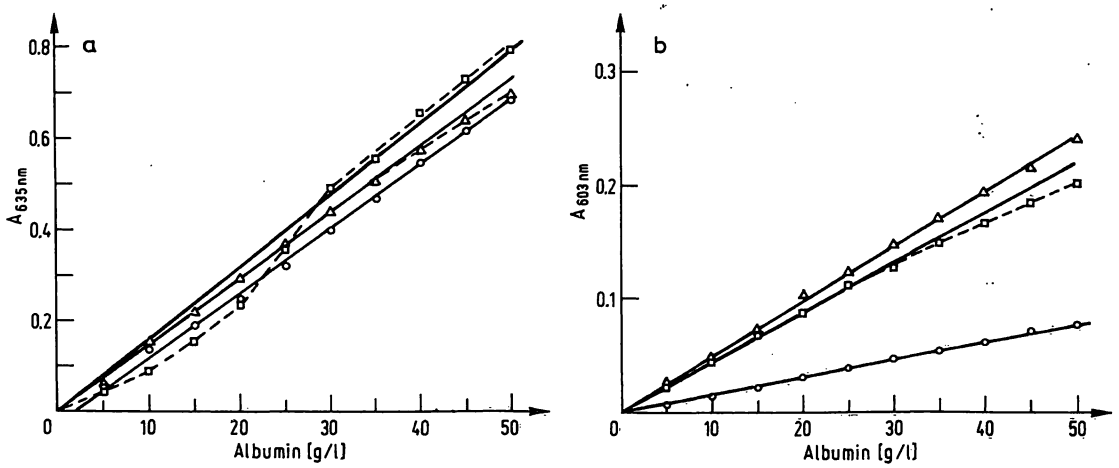

Fig. 1. Standard curves of albumin solutions using both colour methods.

a. Bromocresol green:
- △ Human Albumin (stock solution, 3 ml/l)
- 0 Bovine Albumin (stock solution, 3 ml/l)
- □ Human Albumin (stock solution, 6 ml/l)

b. Bromocresol purple:
- △ Human Albumin (stock solution, 2 ml/l)
- 0 Bovine Albumin (stock solution, 2 ml/l)
- □ Human Albumin (stock solution, 1 ml/l)

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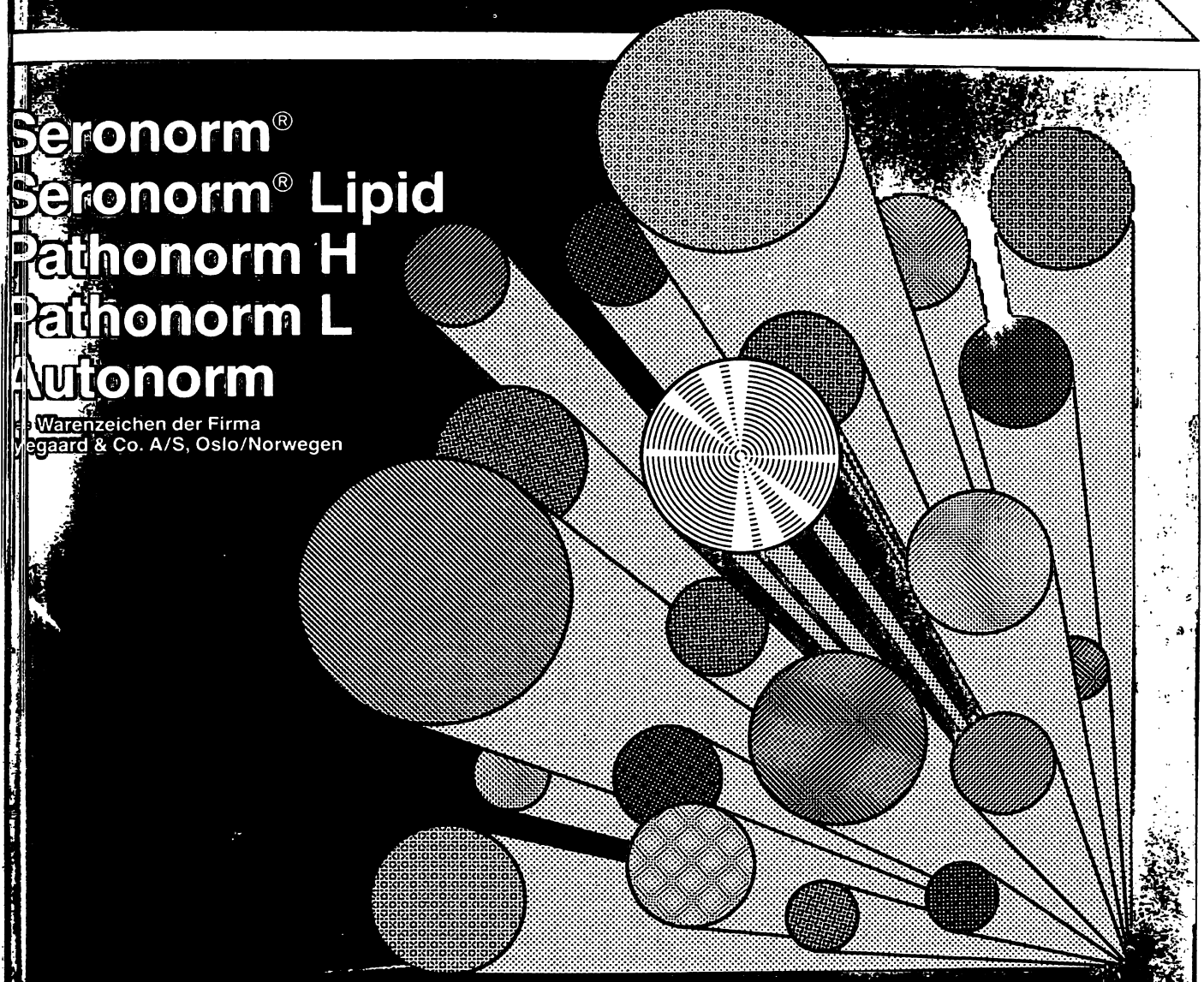

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(bromocresol purple method), it can be seen that the absorption is much less than in the case of human albumin solution. A similar effect was previously reported by *Pinnell & Northam* (4).

The absorption differences between human and bovine albumin solutions are small when measured by the bromocresol green method. Although hardly visible on figure 1 a, the curves show a little "S-character" as estimated by repeated experiments.

The positions of the absorption maxima were found to be at 635 nm for the human albumin bromocresol green complex and at 636 nm for the bovine albumin bromocresol green complex. The corresponding values for the bromocresol purple complexes were found to be at 606 nm and 611 nm.

For the bromocresol purple method we found a better calibration curve if twice (2 ml, 0.04 mol/l bromocresol purple solution) the usual concentration (4) was used. During the preparation of this manuscript *Haythorn* (10) described the same effect. For the bromocresol green procedure, however, this was certainly not the case, and a pronounced "S-character" could be observed (fig. 1 a).

Calibration curves obtained by both colour techniques from a pool serum (containing about 50 g albumin per liter) have the same shape as the standard curves of the human albumin solution. Straight lines were obtained with both methods if a mixture of 50 µl of albumin solution in the working dye reagent solution was further diluted.

With the bromocresol purple method the amount of albumin of a standard human serum (Hoechst-Behring) revealed a value of 63 g/l, and with the bromocresol green method a value of 58 g/l. The recommended and analytical value, however, is 50.9 g/l. Since standard human serum is used as our standard for the immunodiffusion technique, we estimated the value of an albumin solution containing 50 and 60 g/l by this technique. Respective values of 49.3 and 61.8 g/l were found.

In order to investigate whether interfering substances in the serum might be responsible for these high values for the albumin in the Behring serum by both colour methods, we made dilutions of this standard human serum with physiological saline. The absorbance, obtained from the addition of the colour reagents to these solutions, resulted in a straight line.

Addition of a known amount of human albumin to a previously assayed serum revealed that the observed value is equal to the theoretical one.

From the literature it was known that the colour reaction with bromocresol green is not a specific one for albumin determination but that slow reactions with other serum proteins cause an increased absorbance with increasing time (4). With pure human as well as bovine albumin we found no increase of the absorbance either with the bromocresol purple or the bromocresol green procedure.

With some arbitrary human sera, a time dependent increase of the absorbance was observed with the bromocresol green method (about 5% within 30 minutes, and about 7 % after 5 hours). In contrast to the results of other studies (4, 8, 9, 10), a comparable increase was also found with the bromocresol purple method. This increase could be due to a slow unspecific reaction.

We therefore investigated this problem with further electrophoresis experiments. These experiments indicated that other serum proteins are also responsible for a colour development. We observed colours on the positions of the α_2-, β-, and γ-fractions, as indicated in Figure 2.

The colouring was clearly visible if an excess of bromocresol green or bromocresol purple reagent relative to the method as described under "Materials and Methods" was present.

In order to allow a good comparison between the results of the dye method and those of the electrophoresis it is necessary to choose equal concentrations. Two methods were considered:

(1) Dilution of the colour reagent which has the disadvantage that all reactions would be retarded, and
(2) Placement of one strip (after electrophoresis and deacetylating) in 0.6 ml of the dye reagent. We chose the latter possibility. A strip was placed in a little plastic bag (about 7 × 3 cm). Then 0.6 ml of the colour reagent was added and the bag was sealed. For 15 min this bag was rotated in all directions by hand. Next the bag was cut open, and the strip was washed in a buffer (pH about 3). The strip was sealed again and scanned. Colouring of the α-, β-, and γ-globulin positions was clearly visible.

We determined the serum albumin for a number of unselected sera using different techniques: bromocresol purple method, bromocresol green method, M-partigen immunodiffusion plates, and a combination of the determination of total protein and protein scan. We made use of a human albumin standard (50 g/l) and an arbitrary human serum.

The amount of albumin of the latter (39.1 g/l) was estimated by a total protein determination with the biuret reaction and a tenfold electrophoresis followed by scanning.

Table 1 shows the great difference between the results of the different methods.

In quality control we obtained very large deviations from the mean values using the bromocresol purple

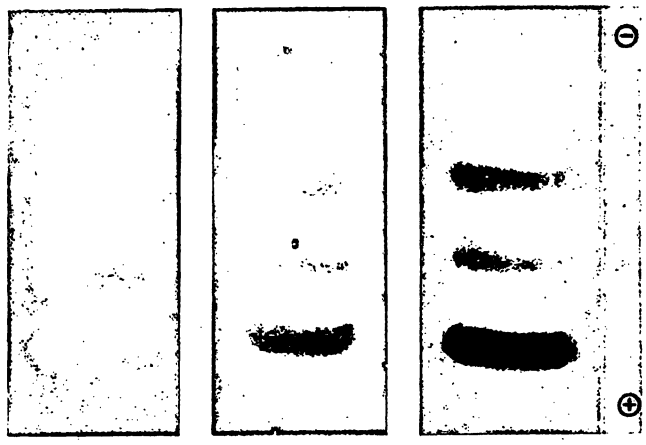

Fig. 2. Protein staining after electrophoresis.
Left : staining with bromocresol green
Middle: staining with bromocresol purple
Right .: staining with amido black

Tab. 1. Determination of serum albumin with bromocresol purple (BCP), bromocresol green (BCG), total protein/protein scan(TP/PS), and M-partigen immunodiffusion (MPID) methods.

a. Standard: Human albumin solution containing 50 g/l.

No.	BCP	BCG	TP/PS	MPID
SHS[1]	59.3	56.0		50.9
SHA[2]	50.0	50.0	50.0	49.3
1	39.4	46.5	40.6	38.3
2	32.5	43.8	41.9	38.4
3	26.5	41.5	36.9	35.3
4	24.0	36.5	33.0	29.7
5	44.0	44.8	45.8	45.4
6	33.0	52.8	47.4	49.2
7	48.3	51.2	45.1	51.0

(1) SHS = Standard Human Serum. Recommended albumin content: 50.9 g/l. This standard was used for the MPID method.

(2) SHA = Standard Human Albumin Solution containing 50 g/l

b. Standard: Human serum containing 39.1 g/l albumin.

No.	BCP	BCG	TP/PS
Standard	39.1	39.1	39.1
1	41.8	40.4	47.0
2	44.8	37.3	43.5
3	44.8	40.0	40.3
4	43.5	43.0	43.3
5	37.5	36.6	22.5
6	36.4	39.3	41.7
7	47.0	41.2	29.5
8	34.4	38.4	41.5
9	43.8	38.6	45.2
10	39.3	39.7	35.6

method when non-human sera were analyzed. The use of the bromocresol green procedure gave a much better agreement with the mean values.

Discussion

Figure 1 shows that the concentration of the dye reagent has an influence upon the shape of the standard curve. In the case of the bromocresol purple method (1 ml, 0.04 mol/l) the curve is flattened at higher albumin concentration (about 30 g/l). This flattening of the curve could be corrected by taking a double bromocresol purple concentration, as compared with the paper of *Pinnell & Northam* (4). For the bromocresol green method doubling of the concentration caused an "S-like" calibration curve. This "S-like" calibration curve is not a result of deviation from *Beer*'s law because a straight line was observed when the solution containing 10 ml of bromocresol green reagent and 50 µl of human albumin standard (50 g/l) was diluted.

We found a very strong difference in absorbance between a bovine and a human albumin standard for the bromocresol purple. Since the absorption maxima between the complexes of human and bovine albumin with bromocresol purple are quite different and since the chemical compositions of human and bovine albumin are not exactly the same (11) we believe that different interactions could be responsible for this effect. Therefore we believe that an earlier explanation of *Pinnell & Northam* (4), who suggested different reactivities between the two types of albumin, is too restricted.

Until now it was believed that bromocresol purple was a specific dye reagent for albumin (4, 8, 9, 10). Our results prove that this statement is not correct.

Our electrophoresis experiments, indicating a colour development with serum proteins in the α_2-, β-, and γ-fractions, agree with this conclusion. Human albumin only gives a colour at the albumin position. It was shown that the double bromocresol purple concentration (4) is not responsible for this phenomenon, because the same results were obtained with the concentrations as used by *Pinnell & Northam* (4).

In contrast to the results of *Webster* (3) and *Louderback* (9), we observed colouring of the γ-globulins in serum with bromocresol green.

Finally a number of methods, including bromocresol green and bromocresol purple were compared (tab. 1). The values of the bromocresol green method tend to be higher than those obtained with the M-partigen immunodiffusion plates. For the bromocresol purple method some values are higher, while others are lower than those obtained by the immunodiffusion method. The higher values could be understood by assuming unspecificity of the reaction, but for the lower values we have no satisfactory explanation.

Although it was known from the literature that the bromocresol purple method underestimated the amount of serum albumin for icteric patients (4, 8) we believe that other factors must also be taken into account to explain these values. The different forms of the calibration curves, obtained with the bromocresol purple and the bromocresol green methods, suggest different reaction mechanisms between the reactions of albumin with these dyes.

In our experiments a human albumin solution (50.0 g/l) was used as a standard. In order to rule out the possibility that a less suitable standard was chosen, we repeated the experiments for the greater part, using a human serum standard (albumin content 39.1 g/l). Table 1 shows that comparable results were obtained.

In the Dutch quality control program our deviations from the means of albumin values can for the greater part be explained by the use of a non-human control serum. Some commercially available non-human control sera were estimated, and showed the same tendency.

If the bromocresol purple method is used, it should be emphasized that very misleading results could be given to the clinic, if the origin of the standard serum is not checked. With the bromocresol purple method a human calibration serum is necessary.

Despite some disadvantages of the bromocresol green method, we believe that for emergency determinations of albumin the bromocresol green method is still preferable to the bromocresol purple method. In other cases the more specific immuno chemical methods are to be preferred.

Very recently *Ingwersen & Raabo* (5) described a modified method of *Doumas* et al (1) for the determination of serum albumin with bromocresol green. We carried out some experiments with their bromocresol green method (5), "our" bromocresol green method (2), and a combination of total protein and protein scan. With *Ingwersen*'s bromocresol green method we obtained lower values than were obtained with "our" bromocresol green method. Comparable values of their bromocresol green method and the total protein/protein scan method were found. Despite some misleading misprints in *Ingwersen & Raabo*'s paper their modification seems to be useful because of better circumstances for the determination of serum albumin with bromocresol green.

In quality control programs using non-human control sera, large deviations from the true albumin value can be obtained when using different dying techniques.

Results can only be compared when the methods are specified.

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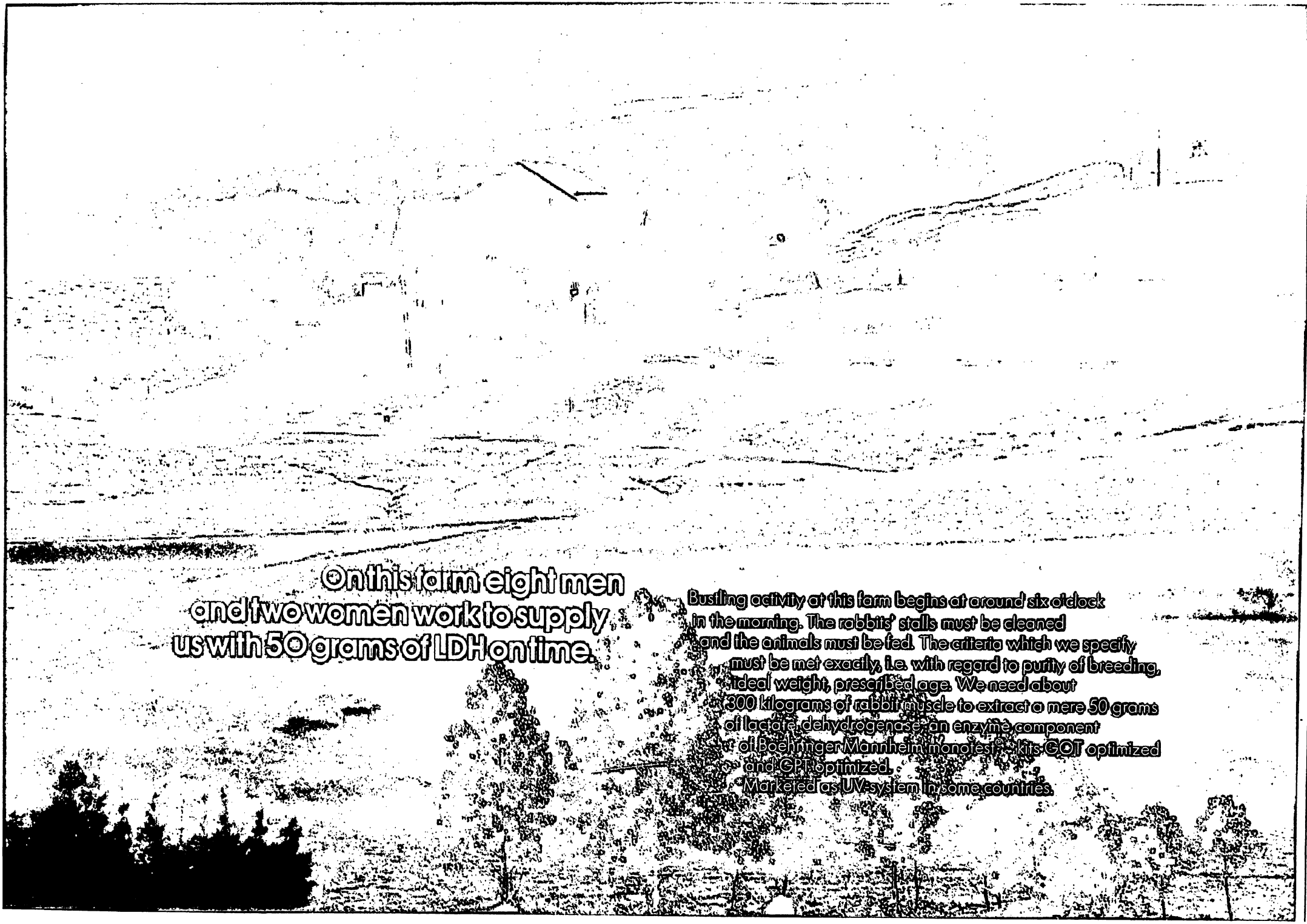
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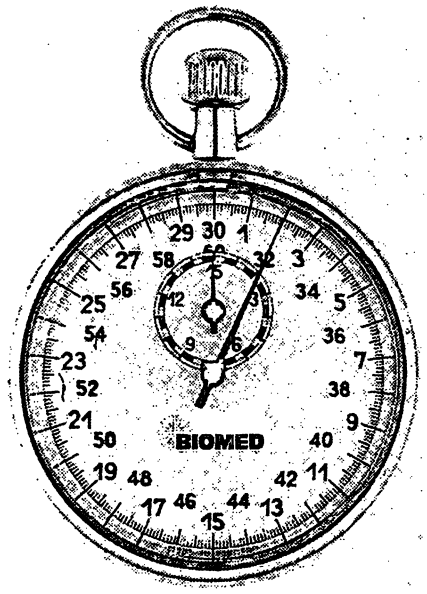

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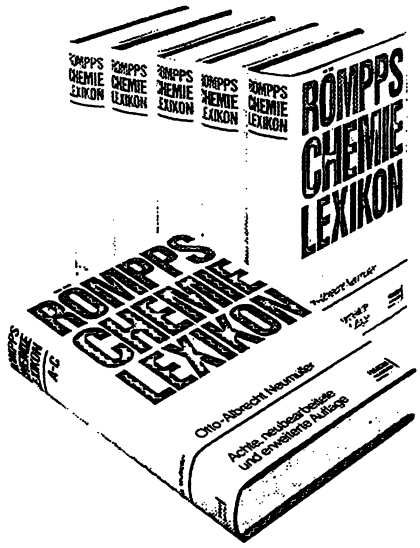

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